# Factors Affecting Oviposition by the Parasitoid *Microplitis croceipes* (Hymenoptera: Braconidae) in an Artificial Substrate

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**ABSTRACT** Several factors that may affect oviposition by *Microplitis croceipes* (Cresson) in an artificial oviposition substrate were investigated. Significantly fewer females oviposited in thin disks of agarose gel than in hemispheres, cylinders, or cubes, although there were no differences in numbers of eggs laid. A higher percentage of females that had oviposited in a *Heliothis zea* (Boddie) larva before testing oviposited in an artificial substrate than did females without oviposition experience. In addition, experienced females laid more eggs than inexperienced females. When the artificial substrate was colored with red, yellow, blue, or green food color, colored substrates elicited more ovipositions than did uncolored substrates, and the number of eggs laid was highest for the blue substrate. The mean number of eggs laid increased also with the food color concentration. The total number of eggs laid increased with the number of substrates per Petri dish. *M. croceipes* preferred hemispherical substrates with a volume of 35–40 $\mu$l, which were similar in height to their actual host instar preferences. A water extract of larval feces did not increase oviposition by *M. croceipes*. Bioassay test criteria and the use of choice tests for ovipositional response of *M. croceipes* are discussed.

**KEY WORDS** Insecta, mass rearing, *Heliothis*, host selection

THE TOBACCO BUDWORM, *Heliothis virescens* (F.), and the cotton bollworm, *H. zea* (Boddie), are major pests of cotton in the U.S. Because of problems associated with control by pesticides (Sparks 1981), emphasis has shifted toward the biological control of these pests. The parasitoid *Microplitis croceipes* (Cresson) is believed to have great potential for the biological control of *Heliothis* species (Knipling & Stadelbacher 1983).

One possibility for enhancing the biological control of *Heliothis* is through the inundative release of parasitoids such as *M. croceipes* (Van den Bosch et al. 1982). However, this method requires an efficient means of parasitoid mass production. Relatively efficient means of rearing *M. croceipes* on *Heliothis* larvae have been described (Powell & Hartley 1987). However, because of host-rearing expenses, these parasitoids are still very costly (about 33 cents per cocoon). One promising method for mass rearing parasitoids is in vitro culture (Greany 1986, Thompson 1986, Greany et al. 1988); however, a simple method for obtaining large numbers of parasitoid eggs is needed. An artificial oviposition substrate (AOS) for stimulating oviposition by *M. croceipes* has been described (Tilden & Ferkovich 1988). Heath et al. (in press) describe the progress in the isolation and identification of the oviposition stimulating kairomone (OSK) for *M. croceipes*.

The purpose of this research was to investigate several factors that may affect the ovipositional behavior of *M. croceipes* toward an AOS and to determine which of these factors may be used to improve egg collecting efficiency.

## Materials and Methods

**Parasitoids.** *Microplitis croceipes* cocoons were purchased from the Delta States Research Center, USDA-ARS, Stoneville, Miss. Cocoons were held for emergence in an environmental chamber maintained at 26 ± 1°C, 70% RH, and a photoperiod of 15:9 (L:D). Males and females were caged together to allow mating and were provided with water and honey. Females were used 3–4 d after adult emergence and except for the oviposition experience experiment, were used without prior exposure to *Heliothis* larvae or artificial oviposition substrates (AOS).

**Oviposition Stimulating Kairomone.** Hemolymph from *Heliothis zea* larvae, which contains an oviposition stimulating kairomone (OSK) (Tilden & Ferkovich 1988), was collected and handled as described by Heath et al. (in press).

**General Experimental Procedure.** AOSs were prepared from agarose (Sigma Scientific, St. Louis)

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dissolved in boiling water to produce a 1% gel solution. For experiments other than the shape experiment, 30 μl of the gel solution was pipetted onto the bottom of a 9-cm plastic Petri dish to form a hemispherical drop; before the drop hardened, a small bubble (about 15 μl) was made on the top of the drop. After hardening, the bubble was broken and 10 μl of hemolymph was pipetted into the concave indentation. The hemolymph was allowed to diffuse throughout the drop for 45–60 min before female wasps were introduced into the dish.

Females were placed individually in Petri dishes containing between 1 and 10 AOSs, depending on the experiment. Petri dishes were then placed in a plastic shoe box (32 by 24 by 10 cm) with wet paper toweling on the bottom to prevent desiccation of the AOSs. The dishes were held in an environmental chamber maintained at 26–27°C. Testing began 2–4 h after the onset of photophase. The percentage of females ovipositing (at least once) in a given treatment, and the number of eggs laid were recorded after 2 or 4 h, or both. One replication of percentage of females ovipositing was generated from five females, and these same five females generated five replications of number of eggs laid.

**Effect of AOS Shape.** To determine if shape affected the ovipositional response of *M. croceipes*, four shapes were tested: hemispherical (about 4.9 mm diameter by 2.4 mm height), cubic (about 3.1 mm on edge), cylindrical (about 1.5 mm diameter by 16.5 mm length), and thin disk (about 0.2 mm height by 13 mm diameter). The cubes were cut from a 3.1-mm thick slab, and the cylinders were extruded from a glass tube (1.5 mm inside diameter). The volume of each shape tested was held constant at 30 μl and 10 μl of hemolymph was added to the agar shape. The four shapes were tested in a choice test (i.e., all four shapes present in a Petri dish). Five females were tested each day, and the entire experiment was repeated 11 times.

**Effect of Oviposition Experience.** To determine whether an oviposition experience with an actual host affected the subsequent response of *M. croceipes* towards the AOS, females were allowed to sting a *Heliothis zea* larva (third instar) about 10 min before being placed in the Petri dish with a hemispherical AOS. Inexperienced females had no prior exposure to either *Heliothis* larvae or to an AOS. Five females of each type were tested each day, and the entire experiment was repeated 10 times.

**Effect of AOS Color.** To determine if color of the AOS affected the ovipositional response, four colors (red, yellow, green, and blue) were compared with an uncolored AOS (actually a light aquamarine color conferred by added host hemolymph). The four colors (with hemolymph) (Sauer's Food Color and Egg Dye, Richmond, Va.) were tested at a concentration of 25 μl food color per 20 ml of a 1% agarose solution (i.e., 0.125% food color by volume). The five treatments were compared in no-choice (i.e., one AOS per dish) and choice (all five in one dish) tests. Five females were tested to each treatment each day, and the entire experiment was repeated 10 times.

To determine whether food colors had OSK activity themselves (i.e., elicited oviposition without hemolymph), the four colors (without hemolymph) were compared with an uncolored AOS with hemolymph. The five treatments were tested in a choice test (i.e., all five in one dish). Five females were tested each day, and the entire experiment was repeated 10 times.

**Effect of Food Color Concentration.** Based on the results of the color experiment, the effects of food color concentration were investigated. Using blue food color, five concentrations were tested: 0.0125, 0.0375, 0.125, 0.375, and 1.25% food color in a 1% agarose solution (by volume). The five concentrations were tested in choice tests (i.e., all five concentrations in one dish). Five females were tested each day, and the entire experiment was repeated 11 times.

**Effect of AOS Density: Number per Dish.** As a result of the previous choice experiments (i.e., shape, color, and concentration), the effect of AOS density was studied. Five densities of hemispherical AOSs per dish were tested: 1, 2, 4, 5, and 10 AOSs per dish. Five females were tested to each density each day, and the entire experiment was repeated 10 times.

**Effect of AOS Size.** Eight sizes of hemispherical AOSs were tested to determine the optimal size: 10- , 15- , 20- , 30- , 40- , 80- , 200- , and 400-μl hemispherical drops. These drops had diameters of 3.4, 3.9, 4.2, 4.9, 5.2, 6.7, 8.1 and 11.5 mm, respectively and heights of 1.7, 1.9, 2.1, 2.4, 2.6, 3.4, 4.6, and 5.8 mm, respectively. Each size drop had a bubble made on top (about one half the drop volume), and the ratio of hemolymph volume to drop volume was held constant at 1:3 to eliminate any concentration effect. Drop sizes were compared in a no-choice test (i.e., one size per dish). Five females were tested to each size each day, and the entire experiment was repeated 10 times.

**Effect of Water Extract of Larval Feces.** The ovipositional response of *M. croceipes* towards a water extract of larval feces as well as its ability to enhance the response to the hemolymph was tested. The three treatments were hemolymph alone, feces extract alone (10 μl of a 0.1 mg/μl water extract of feces from third-instar *Heliothis* that was fed cowpea seedlings), and hemolymph plus feces extract. The three treatments were compared in a no-choice test. Five females were tested to each treatment each day, and the entire experiment was repeated 10 times.

**Statistical Analyses.** Statistical analyses were performed using the Statistical Analysis System (SAS Institute 1985). Analyses of variance were performed on percentage of ovipositing data after angular transformation of percentage and on numbers of eggs laid after square root plus one

**Table 1. Effect of artificial oviposition substrate shape on response of *M. croceipes***

| Test duration | Mean % females ovipositing[a] | Mean no. eggs laid per female[b] |
|---|---|---|
| 2 hours | | |
| Cylinder | 38.2a | 1.7a |
| Hemisphere | 27.3ab | 1.2a |
| Cube | 27.3a | 1.7a |
| Thin disk | 18.2b | 1.2a |
| Total | | 5.8 |
| 4 hours | | |
| Cylinder | 56.4a | 3.1a |
| Hemisphere | 63.6a | 3.0a |
| Cube | 49.1ab | 3.4a |
| Thin disk | 35.5b | 2.9a |
| Total | | 12.4 |

For a given test duration, means within columns without letters in common differ significantly ($P = 0.05$; Duncan's [1955] multiple range test).
[a] $n = 11$.
[b] $n = 55$.

**Table 2. Effect of color of artificial oviposition substrate on ovipositional response of *M. croceipes*[a]**

| Color | Mean % females ovipositing[b] | | Mean no. eggs laid per female[c] | |
|---|---|---|---|---|
| | No-choice test | Choice test | No-choice test | Choice test |
| Blue | 76.0a | 68.0c | 17.8a | 6.5b |
| Red | 78.0a | 66.0c | 13.8a | 5.7ab |
| Yellow | 74.0a | 62.0bc | 12.4a | 5.5ab |
| Green | 66.0a | 58.0ab | 11.6a | 5.8ab |
| Uncolored | 64.0a | 54.0a | 11.4a | 3.8a |
| Total | | | | 27.3 |

[a] Data for 4 hour count. Means within columns without letters in common differ significantly ($P = 0.05$; Duncan's [1955] multiple range test).
[b] $F = 1.29$; df = 4, 36; $P > 0.25$ for no-choice tests and $F = 2.95$; df = 4, 36; $P < 0.05$ for choice tests. $n = 10$.
[c] $F = 6.25$; df = 4, 36; $P < 0.005$ for no-choice tests and $F = 6.17$; df = 4, 36; $P < 0.005$ for choice tests. $n = 50$.

transformation. For the oviposition experience experiment, treatment means were compared using Student's $t$ test. For experiments with more than two qualitative treatments (i.e., shape, color, and feces extract), treatment means were compared using Duncan's (1955) multiple range test. For experiments with levels of a quantitative factor (i.e., color concentration, number per dish, and size), treatment sums of squares were divided into polynomial component sums of squares and reported equations represent significant polynomials in $X$. Significance levels were $P = 0.05$ for all tests.

## Results

**Effect of AOS Shape.** The mean percentage of females ovipositing and the mean number of eggs laid in the four AOS shapes are shown in Table 1. The thin disk AOS elicited the lowest percentage of females ovipositing after 2 and 4 h and was significantly lower than the cylindrical shape after 2 h ($F = 3.94$; df = 3, 30; $P < 0.025$) and significantly lower than the cylindrical and hemispherical shape after 4 h ($F = 5.93$; df = 3, 30; $P < 0.005$). However, the thin disk shape did not elicit a significantly lower mean number of eggs laid than did the other shapes tested after either 2 h ($F = 1.00$; df = 3, 30; $P > 0.25$) or 4 h ($F = 0.44$; df = 3, 30; $P > 0.25$).

**Effect of Oviposition Experience.** A significantly higher percentage of female *M. croceipes* with prior oviposition experience (actual host) oviposited than did inexperienced females after 2 h (78.0 and 54.0, respectively; $t = 4.16$, df = 9, $P < 0.05$) and 4 h (84.0 and 70.0, respectively; $t = 2.63$, df = 9, $P < 0.05$). Experienced females laid significantly more eggs than inexperienced females after 2 h (4.8 and 3.2, respectively; $t = 2.50$, df = 9, $P < 0.05$). After 4 h, however, the difference between the number of eggs laid by experienced and inexperienced females (9.0 and 7.1, respectively) was not significant ($t = 2.22$, df = 9, $P > 0.05$).

**Effect of AOS Color.** The percentage of females ovipositing and the mean number of eggs laid for the color experiment (no-choice and choice tests) are shown in Table 2. For percentage of females ovipositing in the no-choice color test, there were no significant treatment effects. In addition, the contrast for colored versus uncolored was not significant ($F = 2.46$; df = 1, 36; $P > 0.05$). In the choice test, blue and red elicited significantly higher percentage of ovipositions than did either the green or uncolored AOSs. For percentage of females ovipositing in the choice tests, the contrast for colored versus uncolored was significant ($F = 5.64$; df = 1, 36; $P < 0.05$).

The blue AOS also elicited the highest number of eggs laid in the no-choice and choice tests, significantly higher than the uncolored AOS in the choice test. For the number of eggs laid, the colored versus uncolored contrasts were significant for the no-choice ($F = 5.36$; df = 1, 36; $P < 0.05$) and the choice ($F = 5.69$; df = 1, 36; $P < 0.05$ tests). In addition, the females in the choice test laid a total of 27.3 eggs compared with between 11.4 and 17.8 eggs in the no-choice test.

After 4 h, females had oviposited in 38.0% of the uncolored AOSs (with hemolymph added) with a mean of 5.0 eggs per AOS. None of the colored AOSs (without hemolymph) elicited any ovipositions.

**Effect of Food Color Concentration.** Percentage of females ovipositing in the AOSs with various concentrations of food color ranged from 32 to 40 after 2 h and from 52 to 60 after 4 h. There were no significant treatment effects on percentage of females ovipositing after 2 h ($F = 1.00$; df = 4, 40; $P > 0.05$) or 4 h ($F = 1.25$; df = 4, 40; $P > 0.05$). However, mean number of eggs laid ($Y$) increased with food color concentration ($X$) after 2 h ($Y =$

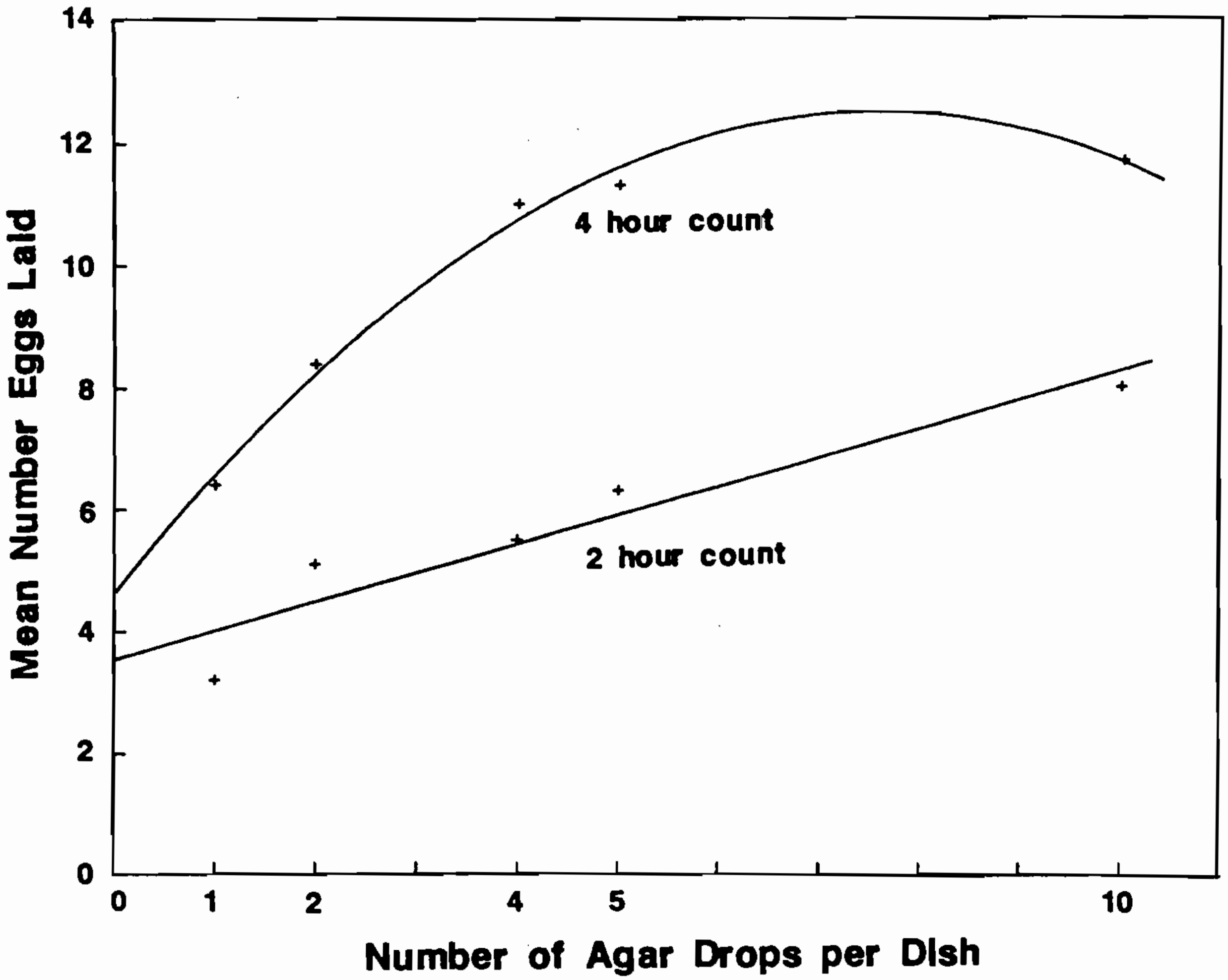


**Fig. 1.** Effect of number of agar drops per dish on number of eggs laid by *M. croceipes* in an artificial substrate.

$1.78 + 0.094X$; $F = 8.25$; df = 1, 40; $P < 0.05$) and 4 h ($Y = 3.48 + 0.18X$; $F = 16.8$; df = 1, 40; $P < 0.05$). In addition, females in this choice test laid an average of 20.7 eggs after 4 h.

**Effect of AOS Density: Number per Dish.** Percentage of females ovipositing in the dishes containing various numbers of AOSs ranged from 62 to 78 after 2 h and from 70 to 92 after 4 h, and there were no significant treatment effects on percentage of females ovipositing after either 2 h ($F = 2.03$; df = 4, 36; $P > 0.05$) or 4 h ($F = 2.54$; df = 4, 36; $P > 0.05$). However, there was a significant linear effect ($F = 1.57$; df = 1, 36; $P < 0.05$) of number of AOSs per dish ($X$) on number of eggs laid ($Y$) after 2 h ($Y = 3.54 + 0.47X$), and the second degree polynomial was significant ($F = 18.2$; df = 2, 36; $P < 0.05$) after 4 h ($Y = 4.60 + 2.09X - 0.14X^2$) (Fig. 1).

**Effect of AOS Size.** The effect of size of AOS on percentage of females ovipositing is shown in Fig. 2a. The third degree polynomial of AOS size ($X = \ln \mu l$) on percentage of ovipositing ($Y$) was significant ($F = 5.72$; df = 3, 63; $P < 0.05$): ($Y = -298.1 + 241.1X - 51.9X^2 + 3.4X^3$). This equation predicts a maximum at 36.8 $\mu$l. The effect of AOS size on mean number of eggs laid is shown in Fig. 2b. The third degree polynomial of AOS size ($X = \ln \mu l$) on the natural log of number of eggs laid was significant ($F = 4.06$; df = 3, 63; $P < 0.05$). Number of eggs laid equals $e^y$, where $Y = -22.7 + 16.8X - 3.7X^2 + 0.25X^3$). This equation predicts a maximum at 40.1 $\mu$l.

**Effect of Water Extract of Larval Feces.** No females oviposited in the AOSs treated with the feces extract only. Forty-eight percent of the females laid eggs in AOSs treated with hemolymph only, and 62.0% laid eggs in hemolymph plus feces extract; these treatments did not differ significantly. In addition, the mean numbers of eggs laid for these two treatments were 5.5 for hemolymph and 6.0 for hemolymph plus feces extract, which were statistically equivalent.

## Discussion

Although the substrate shape had a significant effect on percentage of oviposition, it was a relatively small effect and substrate shape had no significant effect on mean number of eggs laid. Therefore, substrate shape may not be critical to an egg collection procedure. However, ease of making the

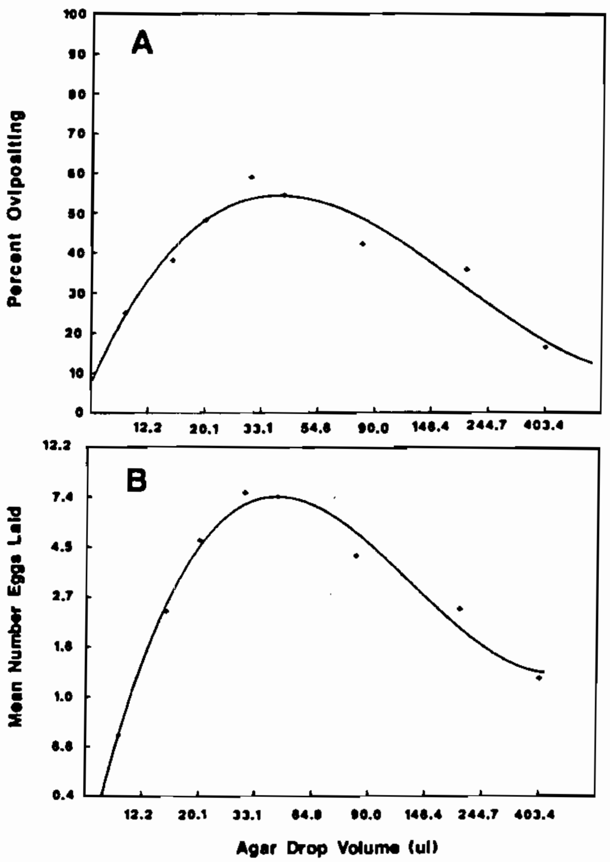


Fig. 2. Effect of agar drop size on (A) percentage of females ovipositing and (B) mean number of eggs laid by *M. croceipes* in an artificial substrate (4-h counts).

various shapes (hemispherical drops easiest, thin disks most difficult) or ease of removing eggs from the various shapes (for subsequent egg development experiments or for in vitro mass rearing) may be more important considerations in shape choice.

Females that have had an oviposition experience with an actual host have a higher percentage of females ovipositing and mean number of eggs laid than inexperienced females. In previous studies, prior experience increased the responsiveness of *M. croceipes* (Drost et al. 1986, Eller et al. 1988a, Lewis & Tumlinson 1988). Surprisingly, in this study, *M. croceipes*' response toward an artificial substrate was increased by prior exposure to a larval host. Although learning cannot be ruled out, this behavioral effect is more likely caused by a change in the female's motivation to oviposit. Interestingly, the difference in responses of inexperienced and experienced females was much smaller (percentage of females ovipositing and number of eggs laid) after 4 h than after 2 h. This suggests that the initially inexperienced females may also exhibit a change in motivational state as a result of their experience with the AOS. This is supported by the result that females with prior experience with AOSs exhibit a higher percentage of females ovipositing and mean number of eggs laid (unpublished data). This method of "prestimulating" *M. croceipes* may be a means to increase the performance of field released parasitoids (Gross et al. 1975).

When the food colors were tested without added hemolymph they did not elicit oviposition, indicating that these colors do not themselves possess OSK activity. Although color has been shown to affect parasitoid host selection behavior (Vinson 1976), the results of the color and food color concentration experiments considered together suggest that the apparent preference for blue may have been a result of that color appearing darkest rather than an actual preference for that color. The darker AOSs may be more apparent to *M. croceipes* resulting in higher encounter rates and subsequently higher oviposition rates. In addition, the colored AOSs provide increased contrast between parasitoid eggs and the AOS, facilitating egg location and subsequent dissection.

In the comparison of numbers of AOSs per dish, percentage of females ovipositing was unaffected although mean number of eggs laid increased with the number of drops per dish. These results are also supported by the results of the no-choice experiments (experience, color, size, and feces extract), which had means of (4 h) 7.1–9.0, 11.4–17.8, 1.2–7.4, and 5.5–6.0, respectively, compared with the choice experiments (shape, color, and food color concentration), which had mean (4 h) dish totals of 12.4, 27.3, and 20.7, respectively. This increase in number of eggs laid is probably a result of an increase in encounter rates in dishes with higher densities of AOSs. The leveling off in the number of eggs laid seen at the higher densities of AOSs (e.g., 5 and 10 AOSs per dish) is probably caused by females reaching some maximum oviposition rate. In addition, although the number of eggs increased with the number of drops per dish, the increase in the number of eggs was less than the increase in the number of drops (i.e., the slope was less than one). Therefore, although more eggs can be obtained by increasing the number of AOSs per Petri dish, the number of AOSs that must be searched increases at a faster rate. Consequently, this method of increasing egg production may not be cost effective.

Host size reportedly affects the host selection behavior of parasitoids (Vinson 1976). *M. croceipes* also exhibits size preferences, preferring to attack third and fourth instar *Heliothis* (Lewis 1970, Hopper & King 1984). In addition, the length of host preferred by *M. croceipes* is reported to be between 6 and 12 mm (Quaintance & Brues 1905, King et al. 1985). These preferences are thought to be a result of smaller larvae being difficult to attack (Lewis 1970) and larger larvae aggressively resisting attack (Hermann & Morrison 1980). The results of the size experiment indicate that *M. croceipes* prefers a hemispherical drop of 35–40 $\mu$l in volume that is about 2.6 mm in height by 5.2 mm in diameter. In an effort to relate AOS size preferences of *M. croceipes* to its actual host size preferences, the heights and lengths of *H. zea* larvae were measured using a dissecting scope with an ocular micrometer. The mean ($n = 5$) heights of newly molted third, fourth, and fifth instars were 1.1, 1.9 and 3.6 mm respectively, and the mean lengths were 6.3, 11.6 and 16.3 mm, respectively. Because the heights of the preferred AOS size and preferred host instars are more similar than are the respective lengths, the reported instar preferences of *M. croceipes* are probably mediated by height, not length.

Although the addition of the water extract of feces to hemolymph elicited a higher percentage of females ovipositing and mean number of eggs laid, neither was significantly higher. These results parallel the results of adding a hexane extract of feces to the hemolymph reported by Heath et al. (1989). Although larval feces are extremely important to the long-range host selection behavior of *M. croceipes* (Eller et al. 1988b, Lewis & Tumlinson 1988), feces had little effect on the ovipositional behavior of *M. croceipes* in the confines of the Petri dish.

Although the no-choice and choice tests gave similar results in the color experiment, there were more significant differences in the choice tests. This suggests that choice tests may be more effective in separating treatments in addition to the inherent efficiency (i.e., fewer insects required per experiment) of choice tests. In addition, except in the comparison of shapes, there were at least as many if not more significant differences using the number of eggs laid data compared with the percentage of females ovipositing data. This suggests that number of eggs laid is a better bioassay test criterion than percentage of females ovipositing. These results are being considered in our current bioassays in the isolation and identification of the oviposition stimulating kairomone.

In summary, these experiments show that various physical and behavioral factors can significantly affect the ovipositional behavior of *M. croceipes* toward an artificial ovipositional substrate. Although the shape of the artificial substrate had only minor effects on the ovipositional response, the effects of shape may be more critical to the subsequent ease of removal of these eggs for further experiments or mass rearing or both. Giving females an ovipositional experience (either with an actual host or an artificial substrate) increases their oviposition rate and could improve egg collection efficiency or be used to 'prestimulate' released parasitoids. The color or concentration, or both, of food dye added to the AOS can also be used to improve egg collection efficiency. Although more AOSs per dish resulted in more eggs being laid, the number of eggs laid does not increase as fast as the number of AOSs. Size preferences of *M. croceipes* indicate that hemispherical drops with a volume of 35–40 $\mu$l and approximately 2.6 mm high are preferred because of their height similarity to preferred *Heliothis* instars. Feces extracts (water or hexane) added to hemolymph did not improve oviposition over hemolymph alone. Using the number of eggs laid as the test criterion is at least as efficient as percentage of females ovipositing, if not more so. In addition, choice tests are more efficient than no-choice tests.

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